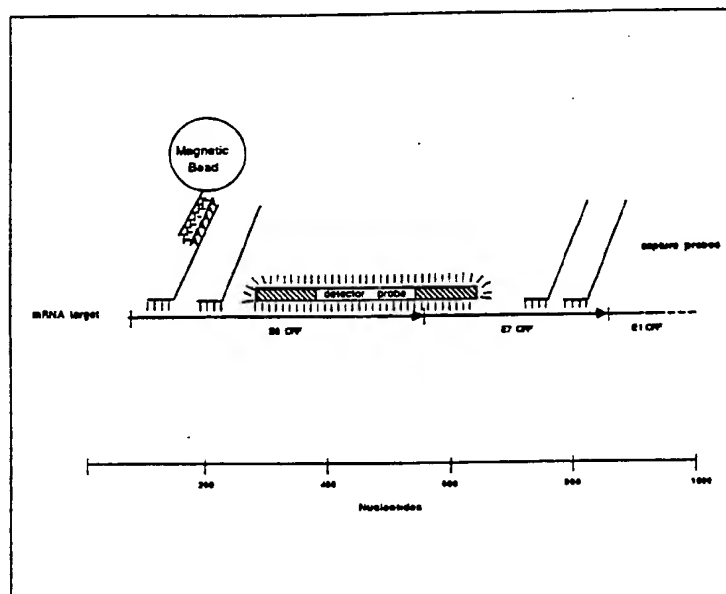




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/70 // C12N 15/37	A1	(11) International Publication Number: WO 91/08312 (43) International Publication Date: 13 June 1991 (13.06.91)
<p>(21) International Application Number: PCT/US90/07057</p> <p>(22) International Filing Date: 3 December 1990 (03.12.90)</p> <p>(30) Priority data: 444,526 1 December 1989 (01.12.89) US</p> <p>(71) Applicant: GENE-TRAK SYSTEMS [US/US]; 31 New York Avenue, Framingham, MA 01701 (US).</p> <p>(72) Inventors: HENDRICKS, David, A. ; 9 Hampstead Road, Jamaica Plain, MA 02130 (US). LANE, David, J. ; 9 Oriole Drive, Milton, MA 01757 (US). RIGBY, Susan ; 23 Deering Avenue, Lexington, MA 02173 (US). PARODOS, Kyriaki ; 30 Royal Crest Drive, Apt. 9, Marlborough, MA 01752 (US).</p>	<p>(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	

(54) Title: DETECTION OF HPV TRANSCRIPTS



(57) Abstract

A method for detecting and/or quantitating specific DNA and/or RNA transcripts of human papillomavirus (HPV) is described. Nucleic acid probes specific for the DNA or the transcripts are used to determine the amount of the DNA or transcripts, and to ascertain the degree of amplification of certain high-oncogenic HPV genes. The method provides an accurate and reliable method for prognosticating serious cervical neoplasias and cancers.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CI	Côte d'Ivoire	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

-1-

DETECTION OF HPV TRANSCRIPTSDescriptionBackground

Epidemiologic data are accumulating which show that the human papillomavirus (HPV) is strongly associated, as a factor or co-factor, with cervical cytologic abnormalities, such as cervical intraepithelial neoplasias (CIN) and carcinoma in situ (CIS). Syrjanen, K.J., In: Papillomaviruses and Human Disease, K. Syrjanen, L. Gissman, and L.G. Koss (Eds.), Springer-Verlag, pp 467-503, (1987). In general, these dysplasias are found in the transition zone of the cervix and are graded from mild to severe (I to III), based on the extent to which neoplastic cells extend from the basal layer to the epithelial surface. Complete replacement of the epithelium by neoplastic cells is termed carcinoma in situ (CIS).

At least 60 types of HPV, isolated from various parts of the human body, have been documented, and more than 20 of these types have been shown to be associated with the genital mucosa. E. M. de Villiers, J. Virol., 63(11):4898-4903 (1989). Although present information strongly supports the close association between HPV and cervical neoplasia, and shows that many individuals clearly have HPV DNA (i.e., infections), transiently and/or latently, it also makes it clear that women in whom HPV is present in cervical cells do not always progress to more serious dysplasia. Syrjanen et al.

-2-

in Cancer Cells, Vol. 5, Cold Spring Harbor, pp. 281-288 (1987); deBrux et al., Bull. Cancer (Paris), 70:410-422 (1983); Mitchell et al., The Lancet, 1:573-575 (1986). Thus, simple detection of HPV DNA in cervical specimens lacks discriminatory predictive value for identification of women at risk for serious disease. A method for assessing HPV activity which can be used to predict progression to serious CIN or CIS is needed.

Summary of the Invention

The present invention relates to a method for detecting and quantitating specific transcripts of HPV and/or HPV DNA in cervical specimens and, in addition, to a method of determining patterns of HPV transcription which are more reliable indicators or predictors of the risk of progression to serious dysplasia or cervical carcinoma in individuals in whom HPV is present than is measurement of total HPV DNA in a sample. The method involves detecting and/or quantitating RNA, DNA or RNA/DNA ratios which are indicative of the presence or absence of high-oncogenic types of HPV. For example, one embodiment of the method involves using nucleic acid probes and/or probe sets comprising DNA or RNA sequences which hybridize to transcripts of the E6 and/or E7 genes of specific HPV types, particularly to HPV types associated with oncogenesis, referred to as high-oncogenic HPV types, (e.g., HPV 16, 18, 31, 33, 35, 39, 45, 51, 52 and 56), but not to low-oncogenic HPV types (e.g., HPV 6 and 11).

The present method can also be used to detect and/or quantitate low-oncogenic HPV types (e.g., HPV 6 and HPV 11), if desired (e.g., as an indication of lower risk of progression to serious CIN or CIS in an individual having an HPV infection than would be the case if high-oncogenic HPV types are present).

The present method is based on hybridization of selected nucleic acid probes for detection and/or quantitation of specific HPV messenger RNAs (mRNAs) or DNA; thus it provides a means for assessing active transcription of the HPV genome.

Type-specific nucleic acid probes and probe sets for HPV are also the subject of the present invention. These probes are of two types: polynucleotide-tailed oligonucleotide capture probes and labelled detector probes (also referred to as labelled riboprobes). Tailed capture probes serve two purposes: 1) they hybridize to at least a portion of an open reading frame (ORF) sequence of an HPV gene or to at least a portion of the encoded transcript of a selected HPV type which is associated with abnormalities, progression to higher grade CIN or development of CIS in cervical epithelial cells; and 2) they link (through the polynucleotide tail) the hybridization complex formed as a result of the method described to a solid support, thus making it possible to separate the hybridization complex from the remainder of the sample. Labelled detector probes are generally single-stranded RNA molecules or single stranded DNA molecules which hybridize to portions of bi- or polycistronic transcripts of genes associated with cervical

-4-

cytologic abnormalities, such as the E6 and/or E7 genes, or to spliced transcripts which contain these ORF's or portions thereof, of selected HPV types; specifically, labelled detector probes form stable hybrids with these transcripts, including spliced transcripts. Although it is possible to use detector probes which hybridize to target sequences which also are recognized by capture probes, it is preferable that they do not. In addition, probes for specific detection of spliced transcripts of the E6 gene of HPV 16, HPV 18, HPV 31, HPV 33 and HPV 35 are the subject of the present invention. Such probes are splice-junction capture probes designed to form stable hybrids only with spliced transcripts of a selected HPV type and they serve as examples of a class of probes which are useful in the present method.

A method for determining prognosis in individuals infected with HPV is also the subject of the present invention. The method is based on the association between HPV transcriptional activity and the risk for development of serious cervical dysplasia or carcinoma. For example, patterns and/or levels of transcription of at least a portion of the E6 and/or E7 genes and spliced downstream sequences of selected high-oncogenic HPV types can be used to predict the progression of HPV infection to serious CIN or CIS. Conversely, patterns and/or levels of transcription of the E6 or E7 genes of low-oncogenic HPV types can be used as an indication of reduced risk of serious CIN or CIS. Thus, the detection of E6 and/or E7 transcripts of selected HPV types in

- 5 -

cervical samples provides a more valuable diagnostic aid than mere detection of HPV DNA.

Certain patterns of HPV transcription can provide a more reliable indicator of risk for progression to serious dysplasia than does detection of total HPV DNA. Low levels of HPV DNA are quite prevalent in women and, thus, the presence of HPV DNA is not a sensitive measure of neoplastic risk. The detection and quantitation of transcripts of genes associated with high-oncogenic or low-oncogenic types of HPV, and/or spliced mRNAs containing these sequences provide a sensitive, accurate, reliable prognosticator of serious dysplasia or cancer.

Brief Description of the Figures

Figure 1 is a schematic representation of the method of the present invention.

Figure 2 is a schematic illustration of the hybridization complex of mRNA targets, d(A)-tailed oligonucleotide capture probes, and radiolabeled detector probes for detection of E6/E7-containing transcripts of HPV.

Figure 3 shows the nucleotide sequences of thirty-three type-specific oligonucleotide probes, which range in length from 32 to 42 nucleotides.

Figure 4 shows nucleotide sequences and target sites of probes for type-specific detection of spliced E6 transcripts (E6*) of HPV 16, HPV 18, HPV 31 and HPV 38. Predicted base pairs between spliced E6* transcripts and specific oligonucleotide probes

-6-

which span the upstream and downstream exons are shown.

Figure 5 is a schematic illustration of capture and detector probes on E6/E7 transcripts of HPV 16, showing the specific arrangement of probes on mRNAs of HPV 16. Splice donor (do) and acceptor (ac) sites in the E6 ORF are indicated.

Figure 6 shows nucleotide sequences of possible base pairs between HPV 16 capture probes 16-3, 16-4, and 16-5 and aligned sequences in the E7 ORF's of HPV 6, HPV 11, HPV 18, HPV 31, HPV 33 and HPV 35. Underlined bases are predicted to form base pairs with the oligonucleotide probe.

Figure 7 is a schematic illustration of the steps for cloning HPV 16 DNA into lambda 47.1 and construction of recombinant molecules for synthesis of detector probes and positive target transcripts. The strategy for engineering a construct for production of the HPV 16 riboprobe is shown on the left side of the figure and the construction of a recombinant molecule for synthesis in vitro of mRNA molecules which contain the E6 and E7 ORF's (i.e., positive target transcripts) is shown on the right side of the figure.

Figure 8 shows the relationship between the concentration of positive target and signal in an HPV 16 assay. Figure 8A shows typical results from the HPV 16 assay using a ^{32}P -labeled detector probe with a specific activity of 5×10^8 cpm/ μg . Figure 8B is a graph showing the linear relationship between the concentration of positive target and the amount of signal.

Detailed Description of the Invention

The present invention is based on an understanding of the close association between HPV and cervical carcinoma; the fact that although many individuals have HPV DNA in cervical cells, they do not progress to more serious dysplasia; and a determination that simple detection of HPV DNA in cervical specimens appears to lack discriminatory predictive value for identification of individuals at risk for progression to serious disease.

In contrast to commonly used techniques for detection of HPV DNA, the present method allows sensitive and quantitative detection of specific HPV mRNA or DNA. The present method makes it possible to assess active transcription of the HPV genome and to determine the associations between HPV transcription patterns and risk for development of cytopathology and/or progression to more serious disease (i.e., higher grade CIN or CIS) in HPV-infected individuals. The following is a brief summary of the assessment of the role of HPV in cervical abnormalities, which establishes the need, and therefore the utility of the present method, a detailed description of the method, a description of HPV-specific nucleic acid probes useful in the method, and discussion of an application of the method in a clinical context for diagnostic and/or therapy-related purposes.

The Role of HPV in Cervical Abnormalities

The role of HPV in cervical abnormalities, which range in severity from mild dysplasia to

- 8 -

complete replacement of the cervical epithelium (CIS), has been studied extensively.

A positive correlation between grade of dysplasia (i.e., CIN I, II or III and CIS) and the presence of HPV has been established. Kurman et al., Am. J. Obstet. Gynecol., 159:293-296 (1988); Koutsky et al., Epidem. Rev., 10:122-163 (1988); deVilliers et al., The Lancet, 2:703-706 (1987). It has been determined that higher grade cytologic abnormalities are more often associated with HPV types 16, 18, 31 and 33 than with low-oncogenic HPV types. HPV types 6, 11, 16 and 18 are found in 80-90% of dysplasias. Koutsky et al., ibid.

However, HPV DNA also has been shown to be present in cervical samples evidencing no abnormality. For example, assays of cervical specimens for HPV DNA of types 6, 11, 16 and 18 by polymerase chain reaction (PCR) from 150 women with no cytologic abnormalities revealed that HPV DNA was present in 70-84% of the women. Young et al., British Med. J., 298(6665):11-14 (1989); Tidy et al., The Lancet, (Feb. 25, 1989) p. 434.

Relatively long-term epidemiologic studies which assess clinical progression from HPV-associated infections (koilocytosis and/or HPV DNA) to CIN I or greater suggest that 8-15% of HPV infections became more dysplastic with time. Syrjanen, K. et al., Papillomaviruses: Cancer Cells, Vol. 5, Cold Spring Harbor, pp 281-288, (1987); de Brux et al., Bull Cancer (Paris), 70:410-422, (1983); Mitchell et al., The Lancet, 1:573-575, (1986); Syrjanen, K. et al., J. Cellular Biochem., Suppl., 13C, p.198

-9-

(1989). Conversely, this suggests that 85-92% of HPV infections do not progress with time. Progression was observed more frequently (about 35%) in individuals infected with HPV 16 than with types 6, 11, 18, 31, and 33 (7.5%-20%). It is unclear, at least, and unlikely, at best, that the presence of HPV DNA alone is a prognostic indicator of progression to serious dysplasias, at least in part because co-factors very likely play roles in disease progression.

Several lines of evidence suggest that expression of the E6 and/or E7 genes of HPV is necessary for oncogenesis. The expression of these genes causes transformation of rat or mouse epithelial or fibroblast cells (Crook et al., Proc. Nat'l. Acad. Sci., 85:8820-8824 (1988); Watanabe and Yoshiike, Int'l. J. Cancer, 41:896-900 (1988)), and, after insertion into a retroviral vector, produces tumors in nude mice. Yutsudo et al., Virology, 166:594-597 (1988). Phelps and co-workers showed that the E7 gene can cooperate with an activated ras oncogene to transform primary baby rat kidney cells and can transactivate a heterologous viral promoter. They also showed that portions of the predicted E7 protein are similar to several of the conserved domains of the adenovirus E1a protein, a transactivating protein which can also cooperate with an activated ras oncogene to transform rat epithelial cells. Furthermore, continued expression of the E7 gene seems to be required for maintenance of the transformed phenotype in cells which have been transformed by HPV 16 and EJ-ras Banks et al., J.

-10-

Cellular Biochem., Suppl. 13C:202 (1988); Crook et al., EMBO Journal, 8(2):513-519 (1989). The E7 protein is localized in the cytoplasm and has been shown, by Western blot analysis, to be the most abundant HPV protein in cell lines containing HPV 16 DNA (CaSki and SiHa) or HPV 18 (HeLa, C4-1, and SW756). Seedorf et al., EMBO J., 6(1):139-144 (1987).

In all HPV-containing cervical carcinomas, and cell lines derived from them, the E6 and E7 genes are intact. Wilczynski et al., Virology, 166:624-627 (1988). Furthermore, transcripts of the E6 and/or E7 gene, but not necessarily other early genes, are consistently found in carcinomas and carcinoma cell lines. Baker et al., J. Virology, 6(4):962-971 (1987); Pater and Pater, Cancer Res., 48:324-328 (1988); Schneider-Gadicke and Schwarz, EMBO Journal, 5(9):2285-2292 (1986); Schwarz et al., In: Papillomaviruses and Human Disease, Springer-Verlag, pp. 443-466 (1987); Shirasawa et al., J. Gen. Virology, 68:583-591 (1987); Smotkin and Wettstein, Proc. Nat'l Acad. Sci., 83:4680-4684 (1986). The evidence suggests that expression of the E6 and/or E7 genes of HPV 16 and 18 are important for cellular transformation and may be important to the development of CIS.

Thus, although presently-available data suggest a strong association between HPV DNA in cervical samples and cervical abnormalities which range in severity from mild dysplasia to CIS, they also make it clear that many, and perhaps most, women in whom

-11-

HPV DNA is present in cervical samples do not progress to dysplasia.

Patterns of transcription, including spliced transcripts, have been examined for several HPV types with high oncogenic potential. Examination of three cell lines containing HPV 18 has shown three patterns of transcription (ascertained by analysis of cDNA clones). Schwarz et al., Nature, 314:111-114 (1985). Pattern 1 contains full length transcripts of the E6 and E7 genes and the 5'-terminal 11 nucleotides (nts) of the E1 RNA (E1 starts at nt 914; splice donor site at nt 925) spliced to 3' cellular sequences. Patterns 2 and 3 contain shortened transcripts of E6 (intron of 182 nts missing between a splice donor site at nts 226-234 and the splice acceptor site at nts 401-412), designated E6*, which are joined to sequences of E7 and either downstream cellular sequences (Pattern 2) or to downstream HPV sequences. (Pattern 3). E6* splice sites also are present in DNA sequences of HPV 16, HPV 31, HPV 33, HPV 35, HPV 43, HPV 52 and HPV 56 but not in DNA of low-oncogenic HPV types (i.e., 6 and 11) Goldsborough et al., Virology, 171:306-311 (1989). Therefore, the E6* transcripts may be related to oncogenic potential of the HPV types. E6/E6* and E7 transcripts are found in HPV 16-containing cells, but they also contain spliced downstream E2-E4 exons, followed by cellular sequences. Two HPV 16 E6* transcripts have been identified in SiHa cells and two cancers. Smotkin et al, J. Virology, 63(3):1441-1447 (1989). Both of them use a common

-12-

donor splice site at nts 224-232 and a splice acceptor site at either nts 399-417 (E6*I) or at nt 516-543 (E6*II). Because translation of E6*I terminates at a greater distance from the E7 translation start signal (AUG) than does translation termination of E6*II, the former transcript may allow more efficient translation of the E7 ORF.

Such transcription patterns described above form the basis for the design of probes and for their use in the method of detection and/or quantitation of specific transcripts of HPV in samples in the present invention. In addition, they provide the basis by which the present method is used for assessing risk of progression of cervical abnormalities. They provide the basis by which the present method is employed to detect and/or quantitate the above-described transcription patterns and/or other transcription patterns for assessing risk of progression of cervical abnormalities to more serious disease. As will be explained and illustrated, the present method is one in which RNA, DNA or the ratio of RNA to DNA of specific HPV types in cervical samples are detected and/or quantitated as a measure of neoplastic risk. Detection and/or quantitation of E6, E7 and spliced transcripts of HPV types associated with oncogenesis or progression of cervical dysplasias as a means of determining neoplastic risk provides a novel tool which can be used not only in a diagnostic context (i.e., to assess presence or absence of risk of progression), but also in a therapeutic context (i.e., as a means by which treatment can be designed and/or monitored).

-13-

Detection of Specific HPV Transcripts by the Present Method

The present method specifically involves detection and/or quantitation of RNA of selected HPV types which are associated with oncogenesis. In particular, the expression of the E6 and/or E7 genes, which can include downstream genes, and their expression relative to other genes is believed to be a prerequisite for HPV-related oncogenesis. In one embodiment of the present method, HPV transcription levels, particularly transcripts of at least a portion of the E6 and/or E7 genes of selected HPV types, are measured. That is, the amount of E6 and/or E7 mRNA present in a sample is detected and quantitated. Bicistronic and/or spliced transcripts of these genes also can be measured.

The Method of the Present Invention

The present invention is a nucleic acid hybridization method which makes use of two types of nucleic acid probes, each of which is complementary in sequence to and capable of hybridizing to at least a region of the RNA transcript of the gene of at least one HPV type associated with oncogenesis or to an HPV gene. In particular, HPV DNA or HPV mRNA transcripts expressing at least a portion of the E6 and/or E7 genes, are detected and/or quantitated using the method.

The method is represented schematically in Figure 1 and is described below as it can be applied to any selected HPV type. It is further described, with reference to a specific HPV type (HPV 16). The

-14-

sample to be assayed by the present method for E6/E7 and other spliced mRNA transcripts will generally be, for example, a cervical scrape, smear, mucus specimen, biopsy or tumor specimen. The sample does not have to be separated, filtered or precultured prior to being assayed by the present method. The sample can be mixed or diluted in a medium, if appropriate, and, if necessary, pretreated with an agent which disrupts cell and molecular structures within the cells. This disruption step frees the nucleic acids to be detected (the target nucleic acids). Cells can be disrupted, for example, using chaotropic agents which disrupt the molecular structure of a cell. That is, the agent denatures the secondary, tertiary and/or quaternary structures of biopolymers, including proteins, nucleic acids and polysaccharides, which are generally found in biological specimens. Examples of chaotropic agents include chaotropic salts (e.g., guanidinium thiocyanate), hydrolytic enzymes (e.g., proteases, RNAases) and compounds that disrupt hydrophobic interactions (e.g., sodium dodecyl sulfate, phenols, dimethylformamide, dimethyl sulfoxide, tetramethyl urea or guanidinium hydrochloride). Physical or mechanical means of disrupting molecular structures, e.g., sonication, can also be used to release nucleic acids. If necessary, nucleic acids present in the cells and released from the cells are also treated to render them available for hybridization with complementary nucleic acid sequences (e.g., by heating to render double stranded sequences single stranded).

-15-

Two types of HPV type-specific probes are then contacted with the sample, either simultaneously or sequentially. One is the HPV type-specific tailed capture probes, which can be free or bound to a solid support. The capture probes are allowed to hybridize with the target transcripts in the sample mixture, forming a capture probe/target transcript complex. Either along with the capture probe or after the capture probe/target transcript complex is formed, labeled detector probes are also contacted with the sample, which is maintained under appropriate conditions (e.g., time, temperature, pH, salt concentration, chaotrope concentration) for hybridization of complementary nucleotide sequences to occur and form a capture probe/target transcript/detector probe complex. The capture probe/target transcript/detector probe complex, which, for convenience, is subsequently referred to as the hybridization complex, is recovered from the sample mixture. This recovery is carried out by adding to the mixture containing hybridization complexes a solid substrate which is coated with a polynucleotide complementary to the tail of the capture probe. The tail of the capture probe present in the hybridization complex hybridizes with the complementary substrate, making it possible to separate the entire hybridization complex from the sample. In the case of pre-hybridized capture probes, the substrate containing the attached probe is removed from the sample mixture. Intact hybridization complexes may be released from the solid substrate by treatments which reversibly disrupt

-16-

base pairings (the capture probe forms fewer base pairs with the solid substrate than do the probes with the target transcript) and complexes may be recaptured on to other solid substrates; the result of this cycling of hybridization complexes is reduction of noise which usually binds non-specifically to the solid substrate.

The quantity of detector probes and, thus, of the specific HPV transcript, present on the separated substrate can then be determined using known techniques, such as scintillation counting, densitometric scans of autoradiographs, fluorescence spectroscopy, and beta emission counters (e.g., Betagen). The method used will depend upon the detector probe label. The amount of signal is related to the amount of target nucleic acids captured, which in turn is proportional to the amount of transcript in the sample. The amount of transcript is related to the level of transcriptional activity of the selected gene (e.g., E6/E7 genes), which activity is indicative of the neoplastic risk. Thus, the method provides a sensitive assay for determining the risk of progression of HPV infection to serious CIN or CIS.

In the embodiment of the present invention in which the occurrence (presence or absence) of selected HPV types is determined or quantitated in order to assess risk of progression to serious cervical dysplasia or cancer in situ, the procedure is as follows: as described above, the presence or absence and/or quantity of one or more selected HPV types is determined. These types can be high-

-17-

oncogenic HPV types, low-oncogenic types or both. The results of this assessment are compared with a previously-established relationship between the HPV types detected or quantitated and risk of progression to serious dysplasia of the cervix or to cervical carcinoma. Such a relationship can be determined by using the previously-described method of detecting and/or quantitating selected HPV types in women whose clinical characteristics (e.g., extent of progression of the condition) is also assessed and related to patterns of HPV DNA present and/or patterns of transcription.

HPV-Specific Nucleic Acid Probes

Nucleic acid probes and probe sets which are specific for HPV genes associated with cervical cytologic abnormalities which often progress to more serious CIN or CIS, are used in the present method. Probes which are particularly useful in the present method are oligonucleotide probes having a nucleotide sequence which is complementary to at least a portion of the E6 and/or E7 ORF, or spliced portions thereof. A nucleotide sequence is complementary to a second or target nucleotide sequence (e.g., the E6 or E7 gene of HPV) if it hybridizes and remains hybridized to the second or target nucleotide sequence under the conditions used in the method.

Two types of probes are used: tailed oligonucleotide "capture probes" and labelled "detector probes" (also referred to as labelled riboprobes). Tailed capture probes serve two purposes: they are complementary to/ hybridize to at least a portion of

-18-

the ORF sequence of a gene of a selected HPV type or of the encoded transcript (mRNA) and they link the hybridization complex (capture probe/target nucleotide sequence/detector probe) to a solid support, thus making it possible to separate the hybridization complex from the remainder of the sample. A schematic representation of the complex of detector probe, target transcript, capture probes and solid support is shown in Figure 2. Labelled detector probes are generally single-stranded RNA or DNA probes which hybridize to portions of the bi- or polycistronic transcripts of genes of selected HPV types; specifically, they hybridize to portions of these genes which can be, but preferably are not also recognized by the capture probes. The selected HPV types are either high-oncogenic or low-oncogenic.

The capture probes are characterized by a polynucleotide tail which is generally formed from a nucleotide homopolymer, such as polydeoxyribadenylate (poly(dA)), polydeoxyribocytidylate (poly(dC)), polydeoxyriboguanylate (poly (dG)), and polydeoxyribothymidylate (poly(dT)). The probe tail is complementary to a polynucleotide sequence which is affixed to a solid support, such as magnetic beads, polystyrene beads and polystyrene dipsticks, allowing the probe to be captured and separated from the test sample.

Probes particularly useful in the present invention as capture probes have the following characteristics:

-19-

1. They are synthesized easily by in vitro transcription (for detector probe) or by chemical means (for oligonucleotide capture probes). Detector probes should be easily labeled, and to high specific activities, during in vitro transcription or chemical synthesis or by post-synthetic modification with either isotopic or non-isotopic markers which can be easily quantitated.
2. They are HPV type-specific. Although HPV 6 sometimes has been associated with carcinomas, it and HPV 11 most often are associated with low risk for progression to high grade dysplasia. Because E6 and E7 genes of HPV 6 and HPV 11 show high sequence conservation, probes to these regions may cross-hybridize; this result would not detract from the efficacy of the assay. Probes which are particularly useful in the present assays are those specific for HPV 16, 18, 31, 33 and 35.
3. They are of sufficient length and nucleotide sequence that they do not dissociate from specific target sequences on viral transcripts in the assay format, and hybridize specifically and quantitatively to transcripts of HPV.
4. They hybridize to selected HPV DNA sequences or transcripts of selected types of HPV. Target transcripts contain at least a portion of the ORF to allow for stable hybridization of the

-20-

detector probes and at least one capture probe. Probes are designed in such a manner that efficiency of hybridization of probes to target transcripts is not diminished by excision of introns from transcripts (e.g., formation of E6*I and E6*II).

5. The integrity of probes used is maintained in the conditions of the assay.

Oligonucleotide probes which are particularly useful as capture probes in the present invention are poly-(dA)-tailed oligonucleotide probes, such as those prepared according to the method described by M.L. Collins in European Patent Application 0265244, filed October 21, 1987, the teachings of which are incorporated herein by reference. Capture probes which are prehybridized to a solid substrate also can be used. Prehybridized probes are described in detail by M.L. Collins et al. in co-pending U.S. patent application number 07/321,728 filed March 10, 1989, the teachings of which are incorporated herein by reference.

The second type of probe used in the present method is a riboprobe or detector probe whose sequence is specific for an HPV transcript or selected HPV DNA, and which generally have a detectable label, such as a radionuclide, or fluorescent indicator. Detector probes are used to quantitate the DNA or mRNA. Detector probes can be prepared by art recognized techniques. Detector probes are generally single stranded RNA probes

-21-

which hybridize to bi- or polycistronic transcripts of a selected HPV gene, such as the E6 or E7 gene. The detector probes are preferably labeled with a radionuclide, such as ^{32}P or ^{125}I , but can be labeled by any means which does not interfere with the ability of the detector probes to hybridize to complementary sequences and which can be detected using available techniques.

The present assay system and method of using it can be incorporated into a kit for clinical use. Such a kit includes a sample processing solution containing a chelating agent, such as EDTA, and an agent for disrupting molecular structures (e.g., membranes) of cells (e.g., a chaotropic agent); the tailed capture probes and labelled detector probes; at least one buffer; an agent for inhibiting RNAase enzymes (to prevent degradation of the target transcripts); a solid support (e.g., magnetic heads or polystyrene substrate) coated with a polynucleotide which is complementary to the capture probe tail; reference or standard nucleic acids to be run simultaneously with the sample as a control; and a wash buffer containing a detergent and an agent (e.g., a chaotropic agent) for disrupting molecular structures of cells. The kit can optionally contain additional wash buffers, a means for detecting the labelled detector probe, one or more elution buffers, amplification or cloning reagents and/or additional positive control samples or negative control sample. Amplification of the target sequences, if desired, can be accomplished, for example, by the technique described by Mullis in

-22-

U.S. Patent 4,683,202. Amplification of the detector probe, after it has been cycled with the hybridization complexes, and/or cloning of the target sequences, can be accomplished, for example, by the method described by Maniatis et al. in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

The invention will now be further illustrated by the following Exemplification.

EXEMPLIFICATION

Design, Preparation, and Physical Description of Probes

HPV type-specific oligonucleotide capture probes, about 40 nucleotides long are shown in Figure 3. The Wordsearch/Segments Programs of the University of Wisconsin Suite was used to identify regions of HPV genomic, sense strands (i.e., RNA sense) which might hybridize to these 33 oligonucleotide probes, and results of these analyses are shown in the Table.

- 23 -

Table. Predicted Number of Base Pairs
Between Oligonucleotide Probes and
Discreet Regions of HPV Genomes

HPV Type	Probe No.	Probe Length	ORF	Nts Recognized By Probe	6	11	16	18	31	33	35
6	6-1	40	E6	111-150	40	34	<20	NF	24/4	<20	NF
	6-2	39	E6	167-205	39	30	23/8	NF	25/1	22/6	NF
	6-3	32	E7	644-675	32	25	23/6	<20	<20	NF	NF
	6-4	36	E7	715-750	36	30	21/4	22/1	<20	NF	NF
	6-5	36	E7	761-796	36	21/10	NF	NF	22	NF	NF
11	11-1	40	E6	112-151	34	40	<20	<20	<20	NF	NF
	11-2	40	E6	152-191	33	40	NF	NF	22/1	22/8	NF
	11-3	40	E7	561-600	36	40	<20	<20	24/2	20/3	NF
	11-4	40	E7	669-708	37	40	NF	23/4	<20	<20	NF
	11-5	36	E7	715-750	30	36	<20	23/8	<20	NF	NF
	11-6	40	E7	755-794	31	40	20	25/2	<20	NF	NF
16	16-1	42	E6	96-133	<20	23	38	NF	<20	NF	22
	16-2	40	E6	150-189	<20	NF	40	<20	22/3	20/1	NF
	16-3	40	E7	701-740	<20	<20	40	21/4	32	29	30
	16-4	40	E7	747-786	<20	<20	40	21/4	<20	<20	NF
	16-5	40	E7	787-826	26/1	20	40	<20	32	22/2	NF
18	18-1	38	E6	103-140	NF	NF	<20	40	<20	NF	NF
	18-2	40	E6	157-196	21/3	<20	NF	40	23/3	<20	NF
	18-3	41	E7	627-667	NF	NF	<20	40	23/2	20/4	NF
	18-4	38	E7	752-789	<20	<20	<20	38	<20	<20	NF
	18-5	40	E7	796-835	25	<20	27/3	40	<20	NF	NF
	18-6	40	E7	836-875	<20	<20	NF	40	20	NF	NF
31	31-1	40	E6	103-142	23	24/4	NF	23/1	40	NF	NF
	31-2	40	E6	145-183	<20	<20	<20	<20	40	<20	NF
	31-3	37	E7	721-757	NF	<20	21/2	NF	37	<20	NF
	31-4	40	E7	763-802	<20	NF	<20	NF	40	NF	31
	31-5	39	E7	815-853	NF	NF	28	<20	39	20	28
33	33-1	40	E6	124-163	NF	<20	22	21/1	26	40	24
	33-2	41	E6	164-204	NF	20/2	<20	20/2	<20	40	22/2
	33-3	40	E7	602-641	<20	30/3	30	<20	31	40	33
	33-4	40	E7	713-752	<20	24/8	NF	NF	26/3	40	NF
	33-5	37	E7	756-792	20/4	<20	<20	NF	23/3	37	NF
	33-6	40	E7	798-837	27/6	24/2	22/6	<20	21/2	40	25/2
35	35-1	39	E6	111-149	NF	NF	22	NF	NF	20	39
	35-2	41	E6	153-193	NF	NF	NF	NF	NF	26	41
	35-3	39	E7	740-778	NF	NF	NF	NF	NF	NF	39
	35-4	39	E7	780-818	NF	NF	32	NF	30	NF	39
	35-5	39	E7	829-867	NF	NF	31	NF	30	NF	39

-24-

Numbers in the Table refer to the base pairs in a discreet region of the viral genome which are predicted, from computer analyses (algorithm of Wilbur and Lipman; P.N.A.S. 80:726-739, 1983)), to pair with portions of the oligonucleotides. Numbers after slash marks (/) indicate the gaps that would be needed in the oligonucleotide or target sequence to allow for the number of base pairs. For each HPV type, the nucleotide sequences (GenBank designations) that are covered by each type-specific oligonucleotide are shown. In some cases, the computer program did not find any likely regions of significant base pairing between the oligonucleotide sequences and genomic sequences; these cases are noted "not found" (NF). The oligonucleotides in Table 1 are unlikely to form stable hybrids with other types of HPV, therefore, they are considered to be type-specific.

Design of Exon-Specific Capture Probes for HPV 16, HPV 18, HPV 31, HPV 33 and HPV 35

Splice junction sequences in the E6 ORF have been identified in several oncogenic types of HPV, including HPV 16, HPV 18, HPV 31, HPV 33, and HPV 35 but they are not present in the E6 ORF's of HPV 6 and HPV 11. These splice donor and acceptor sites are utilized in HPV 16 and HPV 18, as is demonstrated by the presence of spliced mRNA's (determined by sequencing cDNA's) in carcinoma cell lines. The presence of spliced transcripts can be used as predictors of progression to more serious disease. Thus, splice-junction capture probes which

-25-

are predicted to form stable hybrids only with spliced transcripts were designed. Approximately 20 nucleotides of each capture probe hybridize to the 3' end of the upstream exon and the remaining 20 nucleotides hybridize to the 5' end of the downstream exon. Sequences of these probes and their target sites on transcripts are shown in Figure 4. Analyses of the alignment (algorithm of Wilbur and Lipman) of splice-junction probes and the genomes of HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, and HPV 33 revealed that each probe is predicted to hybridize only to the transcripts of the HPV type for which the probe was designed. Different probes were designed for detection of each of the two E6 spliced mRNA's (E6*I and E6*II) of HPV 16.

Design of HPV 16-Specific Capture Probes and Arrangement of Probes in the HPV 16 Assay

Ten HPV 16-specific oligonucleotide probes (about 40 nucleotides long) were designed and tested in the assay, described below. Three of these probes, designated 16-3, 16-4, and 16-5, were adapted for use in the assay because they afforded high sensitivity and specificity. The placement of these three probes with respect to the RNA target and the detector probes is shown in Figure 5. Capture probe 16-3, the probe closest to the detector probe, hybridizes to target sequences 46 nucleotides beyond the 3' end of the hybridization site covered by the detector riboprobe. Therefore, after hybridization of the detector probe to mRNA, only a short portion remains single stranded (i.e.,

-26-

not covered by capture or detector probes), thus minimizing the chances for degradation by RNases.

The alignment of these capture probes with sequences in HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, HPV 33 and HPV 35 is shown in Figure 6. Inspection of potential base pairs between these probes and the other genital HPV types, an alignment that was made by visual and computer analyses, reveals that base mismatches (not underlined) are numerous and approximately evenly distributed along the capture probes. As a result, these hybrids would be very unstable.

The 3' ends of capture probes are extended by sequential addition of d(A) residues with terminal transferase such that the final poly-d(A) "tail" is 150-200 nucleotide long, the minimum length that will allow unencumbered capture of the hybridization complex to d(T)-modified magnetic beads.

Design and Preparation of HPV 16-Specific Detector Riboprobe and Target RNA

HPV 16 DNA was cloned from CaSki cells, known to contain HPV 16 genomes (Baker et al., J. Virol., 61(4): 962-971, 1987), into lambda 47.1 from which it was subcloned into the pT7/T3 α -18 plasmid (Bethesda Research Laboratory). A summary of the cloning/subcloning scheme is shown in Figure 7. A 630 nucleotide Dde I fragment (nucleotides 25-655; Seedorf et al., Virol., 145:181-185, 1985) was excised, end-filled, and subcloned into Sma I-restricted pT7/T3 α -18 plasmid. Sequence analysis, obtained by double or triple reads in most cases,

-27-

revealed that the insert contained only nts 25-655 of the HPV 16 genome. In vitro transcription with T7 polymerase on this construct, linearized with Ssp I restriction endonuclease, results in a single product with a homogeneous electrophoretic mobility and an apparent size of 0.5 Kb, that should hybridize to the 3' three-fourths of the E6 ORF and the 5' third of the E7 ORF. The detector probe contains, in addition to 471 nucleotides of the HPV 16 sequence, 22 additional nucleotides of the plasmid sequence on its 5' end, a result of transcription initiation on the T7 promoter and procession through a portion of the multiple cloning site in the vector. These additional 5' nucleotides will not interfere with hybridization of the capture probes, the most proximal being 46 nucleotides away. The detector probe, after hybridization to target mRNA, should extend about 40 nucleotides 5' to the splice donor site at nucleotides 224-232 (Figure 5); this hybrid is predicted to be very stable. Excision of either of the E6 introns would result in mRNA molecules that base pair with either 162 or 288 nucleotides of the detector probes. These hybrids should be stable in the assay. Following the manufacturers' instructions for synthesis of transcripts with T7 polymerase, ³²P-labeled riboprobes with specific activities of 5×10^8 cpm/ μ g are produced. Placement of the detector probe on E6/E7 transcripts was designed such that E6/E7 target sequences flanking the hybridization site of the detector riboprobe would be available for hybridization to capture probes.

-28-

The sensitivity of the HPV 16 assay was also determined. For this purpose it was important to produce, by in vitro transcription, well characterized positive targets (i.e., mRNA-like molecules which contain the ORF's of the E6 and E7 genes). Transcription from the T3 promoter in the construct which contained full length genomic HPV 16, revealed a putative transcription terminator just upstream from the E6 and E7 genes (nucleotides 7400-7600 in the non-coding region). Visual inspection of the sequences in this region, which lies just downstream from a polyadenylation signal, suggests that transcription of plus-sense RNA from this portion of the genome would produce RNA's which have numerous stretches of U residues, characteristics of transcription terminator; therefore, a 1.3 Kb Sph I fragment which includes about half of the region rich in termination sequences and a few sequences in the vector was deleted. Transcription from the resulting construct (linearized with Spe I) with T3 polymerase yields one expected major transcript of 1.9 Kb as well as a shorter minor RNA species (about 600 nucleotides shorter than the major one). The 1.9 Kb transcript was used as a positive target in the assay.

Design and Performance of the HPV 16 Assay

Preparation of samples and procedures for performance of the assay are the same as those described by M. Collins in European Patent Application, Number 0265244, the teachings of which are incorporated herein by reference. In brief,

- 29 -

cells from cervical specimens (e.g., cervical lavage, scrapes, biopsies, and tumors) are mixed with sample processing buffer (SPB), containing 5M guanidinium isothiocyanate (GuSCN) and 0.1M ethylenediaminetetraacetic acid (EDTA) in order to solubilize the cells and sample matrix and to inhibit RNases. Samples are diluted such that the final concentration of GuSCN is 2.5M and incubated with 67 ng/ml of each capture probe and 1.4×10^7 cpm/ml of detector probe at 37° for 2-18 hours. Then, d(T)-coated magnetic beads are added (final concentration of 0.125% solids) and the resulting mixture is incubated at 37° for 15 minutes; d(A)-tailed capture probes hybridize to the d(T)-coated magnetic beads during this period. Wash buffer (0.5 M GuSCN, 40 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% sarkosyl, 0.2% bovine serum albumin, and 0.1% antifoam) is added to the mixture and the magnetic beads then are concentrated on the walls of the tubes by a magnetic field. The supernatant fluid is aspirated. Beads are washed two additional times with wash buffer, following the procedure just described, and then re-suspended in a buffer containing 3.25M GuSCN, 100mM Tris-HCl, pH 7.8, 65mM EDTA, 0.5% BSA, and 0.5% sarkosyl. Following incubation of the suspension at 37° for 10 minutes, nucleic acid complexes should be released from the beads due to disruption of the A-T bonds between the poly-d(A) tails of the capture probes and the oligo-d(T) chains of the magnetic beads. After concentration of the magnetic beads on the tube walls by a magnetic field, the release buffer and

-30-

nucleic acid complexes are transferred to another tube and hybridization complexes are captured on a second set of magnetic beads, following the procedure just described. This capture, release, and re-capture of the nucleic acid complexes on magnetic beads is called reversible target capture.

A second round of reversible target capture is performed and nucleic acid complexes in the release buffer are captured on a third set of beads. After the beads are washed once, they are resuspended in wash buffer and an aliquot of the suspension is applied to a nitrocellulose membrane. Radioactivity can be detected by exposure of the membrane to film or by quantitation of counts on a beta emission blot analyzer (e.g., Betagen). Exposure of the film may be quantitated on a densitometer. Alternatively, fluorescein-labeled detector oligonucleotides or riboprobes may be used in the assay.

Efforts were made to quantitate the sensitivity of the HPV 16 assay using in vitro-generated positive target transcripts. Examination of the X-ray film revealed (Figure 8A) that 500 fg of target (500,000 molecules) could be visualized and that a signal was not detected if target RNA was omitted; no signal was detected if lysates from 200,000 HeLa cells (which contain HPV 18 sequences) are used in the assay. A standard curve, produced by plotting radioactivity on beads vs. amount of positive target RNA, is a straight line for RNA target values of 100 fg to 100 pg (Figure 8B).

-31-

CLAIMS

1. A method of detecting in a sample obtained from an individual at least one high-oncogenic type of HPV, comprising the steps of:
 - a) rendering nucleic acids present in the sample available for hybridization with complementary nucleotide sequences;
 - b) combining the product of step (a) with at least one nucleic acid probe having a nucleotide sequence complementary to a gene of a high-oncogenic type HPV, wherein said gene is selected from the group consisting of the E6 gene, the E7 gene and spliced transcripts of the E6 or E7 gene, or both, under conditions appropriate for hybridization of complementary nucleotide sequences to occur; and
 - c) detecting hybridization of the complementary nucleotide sequences.
2. The method of Claim 1 wherein the high-oncogenic type HPV is selected from the group consisting of HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52 and HPV 56.
3. The method of Claim 2 further comprising quantitating at least one high-oncogenic type HPV, wherein in step (b), the extent to which hybridization of complementary nucleotide sequences occurs is determined.

- 32 -

4. The method of Claim 1 further comprising combining the product of step (a) with a set of nucleic acid probes, the set comprising at least two different nucleic acid probes, each of said probes having a nucleotide sequence complementary to a different selected gene or transcript of a high-oncogenic type HPV.
5. The method of Claim 4 further comprising quantitating in step (c) the extent to which hybridization of each of the nucleotide sequences occurs.
6. A method of detecting, in a cervical sample, a transcript of at least one high-oncogenic type HPV, comprising the steps of:
 - a) combining the cervical sample, previously treated to render nucleic acids present in the sample available for hybridization with complementary nucleotide sequences, with at least one nucleic acid probe having a nucleotide sequence complementary to the E6 gene, E7 gene or spliced transcripts of the E6 or E7 gene, or both, of a high-oncogenic type HPV under conditions appropriate for hybridization of complementary nucleotide sequences to occur; and
 - b) detecting hybridization of the complementary nucleotide sequences.

-33-

7. A method for detecting, in a cervical sample, a target nucleotide sequence which is the transcript of a gene of at least one selected high-oncogenic type HPV, comprising the steps of:
- a) treating the cervical sample to render nucleic acids present in the sample available for hybridization with complementary nucleotide sequences;
 - b) contacting the product of step (a) with:
 - 1) at least one tailed capture probe which a) is complementary to a transcript of at least a portion of the E6 or E7 gene, the gene of the selected high-oncogenic type HPV; and b) has a polynucleotide tail; and
 - 2) a labeled detector probe which is complementary to a different portion of the transcript of the E6 or E7 gene of the selected high-oncogenic type HPV, under conditions appropriate for hybridization of complementary nucleotide sequences to occur, thereby forming a capture probe/ target nucleotide sequence/ detector probe hybridization complex;
 - c) contacting the product of step (b) with a solid support having affixed thereto a polynucleotide which is complementary to the polynucleotide tail of the capture probe present in the hybridization complex, under conditions appropriate for

-34-

hybridization of complementary nucleotide sequences to occur, thereby forming a hybridization complex/solid support complex;

- d) separating the hybridization complex/solid support complex from the product of step (c) by removing the solid support; and
- e) detecting the hybridization complex/solid support complex, the presence of which is indicative of the presence in the cervical sample of the target nucleotide sequence.

- 8. The method of Claim 7 wherein the target nucleotide sequence comprises a nucleotide sequence selected from the group consisting of: a transcript of the E6 gene of a high-oncogenic type HPV gene; a spliced transcript of the E6 gene of a high-oncogenic type HPV gene; a transcript of the E7 gene of a high-oncogenic type HPV gene; and a combination thereof.
- 9. The method of Claim 8 wherein the high-oncogenic type HPV is selected from the group consisting of: HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52 and HPV 56.
- 10. The method of Claim 9 wherein the target nucleotide sequence further comprises HPV nucleotide sequences downstream of the E7 gene.

-35-

11. The method of Claim 9 wherein the capture probe is selected from the group consisting of:
probes 16-1, 16-2, 16-3, 16-4, 16-5, 18-1, 18-2, 18-3, 18-4, 18-5, 18-6, 31-1, 31-2, 31-3, 31-4, 31-5, 33-1, 33-2, 33-3, 33-4, 33-5 and 33-6, 35-1, 35-2, 35-3, 35-4 and 35-5, as represented in Figure 3.
12. The method of Claim 9 wherein the capture probe is complementary to a spliced transcript of the E6 gene of a high-oncogenic type HPV, said probe selected from the group consisting of:
ExHPV16E6*I, ExHPV16E6*II, ExHPV18E6*, ExHPV31E6*, ExHPV33E6*, and ExHPV35E6* as represented in Figure 4.
13. The method of Claim 9 wherein the polynucleotide tail of the capture probe is poly d(A) or poly d(T).
14. The method of Claim 9 wherein the detector probe is labeled with a radionuclide.
15. The method of Claim 14 wherein the radionuclide is ^{32}P or ^{125}I .
16. The method of Claim 9 wherein the solid support is selected from the group consisting of:
paramagnetic beads, polystyrene beads and polystyrene dipsticks.
17. The method of Claim 16 wherein the solid support has affixed thereto a polynucleotide

-36-

selected from the group consisting of poly d(T) and poly d(A).

18. The method of Claim 17 wherein the capture probe is prehybridized with the polynucleotide affixed to the solid substrate.
19. The method of Claim 7 further comprising in step (b) contacting the product of step (a) with a set of nucleic acid probes, the set comprising at least two different nucleic acid probes, each of said probes being a nucleotide sequence complementary to all or a portion of a selected gene of a high-oncogenic type HPV.
20. The method of Claim 7 further comprising quantitating the target nucleotide sequence, wherein in step (f), the extent to which hybridization of complementary nucleotide sequences occurs is determined.
21. A kit for detecting or quantitating, in a cervical sample, a target nucleotide sequence of at least one high-oncogenic type HPV, the target nucleotide sequence being either DNA or RNA, comprising:
 - a) a chelating agent;
 - b) an agent for disrupting molecular structures in cells;
 - c) at least one capture probe which a) is complementary to the target nucleotide sequence and b) has apolynucleotide tail;

-37-

- d) at least one detector probe which is a nucleotide sequence complementary to at least a portion of the target nucleotide sequence;
 - e) at least one buffer;
 - f) an agent for inhibiting RNAase enzymes;
 - g) a solid support having affixed thereto a polynucleotide complementary to the polynucleotide tail of the capture probe;
 - h) reference nucleic acids; and
 - i) a detergent.
22. The kit of Claim 21 wherein the chelating agent is EDTA or DTPA.
23. The kit of Claim 22 wherein the agent for disrupting molecular structures in cells is a chaotropic agent.
24. The kit of Claim 23 wherein the polynucleotide tail of the oligonucleotide capture probes is a d(A) tail or d(T) tail.
25. The kit of Claim 24 wherein the detector probe is labelled.
26. The kit of Claim 25 wherein the solid support is selected from the group consisting of magnetic beads, polystyrene beads and polystyrene dipsticks.

-38-

27. A method of determining the risk of progression to serious dysplasia of the cervix or to cervical carcinoma in a female in whom HPV is present in the cervix, comprising detecting, in a cervical sample, at least one target nucleotide sequence which is all or a portion of a gene of an HPV type or a transcript thereof selected from the group consisting of the E6 gene of a high oncogenic type of HPV, the E7 gene of a high oncogenic type HPV, a spliced transcript of the E6 gene or the E7 gene, or both, of a high oncogenic type of HPV and a combination thereof, and comparing the results with the patterns of transcripts which are associated with high grade dysplasia or carcinoma in situ, or with the risk of progression to serious dysplasia of the cervix or to cervical carcinoma.
28. The method of Claim 27 wherein the high-oncogenic type HPV is selected from the group consisting of: HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52 and HPV 56.
29. The method of Claim 27 further comprising determining the quantity of the target nucleotide sequence in the cervical sample and comparing the quantity of target nucleotide sequence in the sample with the patterns of transcripts which are associated with high grade dysplasia or carcinoma in situ or with

-39-

risk of progression to serious dysplasia of the cervix or to cervical carcinoma.

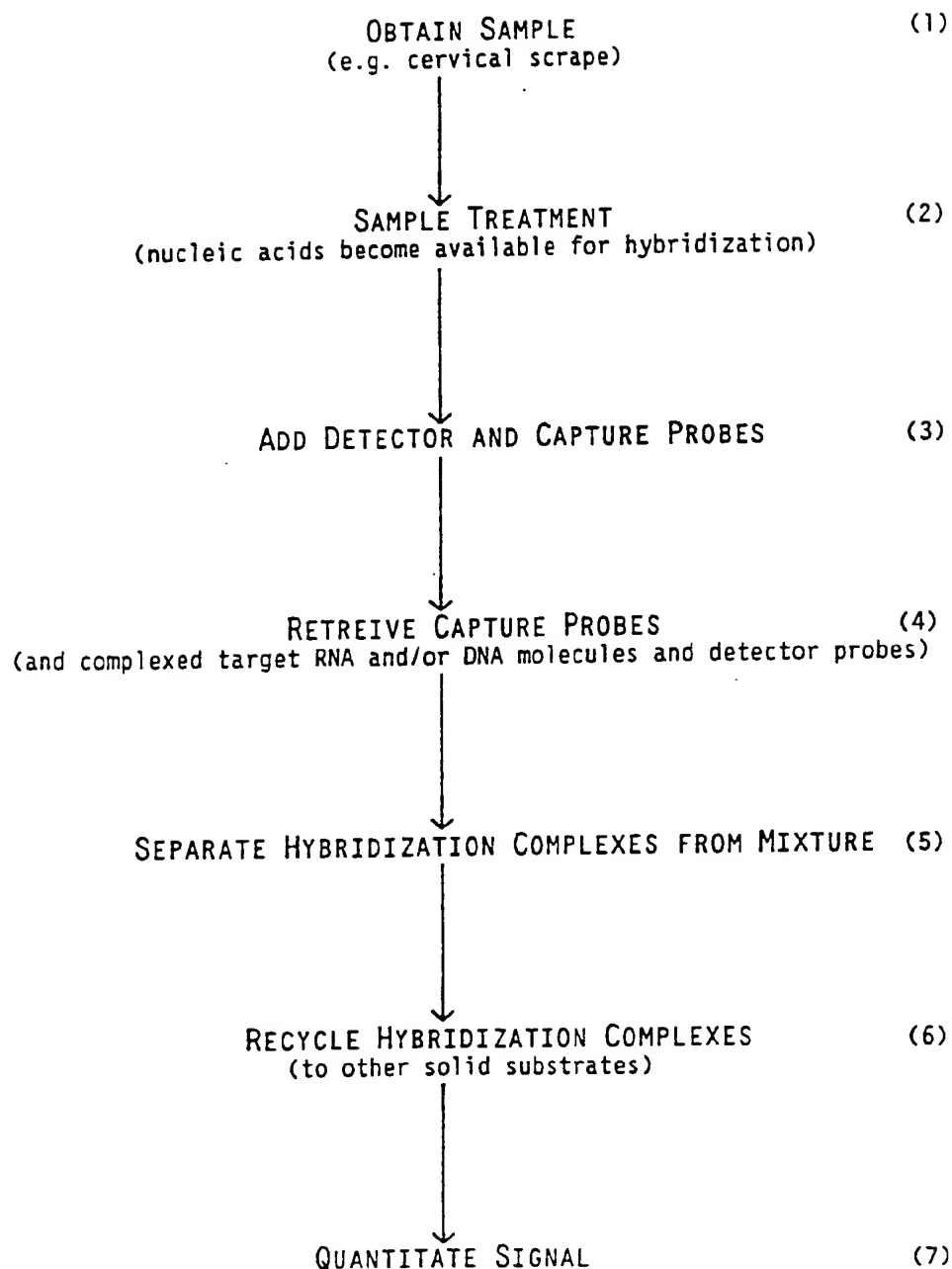
30. The method of Claim 29 further comprising detecting in a cervical sample from the female at least two different target nucleotide sequences, all of said target nucleotide sequences being either DNA of a high-oncogenic type HPV or a transcript thereof, but not a mixture thereof.
31. A nucleic acid probe comprising a nucleotide sequence selected from the group consisting of nucleotide sequences complementary to the nucleotide sequence of at least a region of the E6 gene of an high-oncogenic type HPV and nucleotide sequences complementary to the nucleotide sequence of at least a region of the E7 gene of high-oncogenic type HPV.
32. A nucleic acid probe of Claim 31 wherein the nucleotide sequence comprises at least a portion of a nucleotide sequence selected from the group consisting of the nucleotide sequences of probes 16-1, 16-2, 16-3, 16-4, 16-5, 18-1, 18-2, 18-3, 18-4, 18-5, 18-6, 31-1, 31-2, 31-3, 31-4, 31-5, 33-1, 33-2, 33-3, 33-4, 33-5, 33-6, 35-1, 35-2, 35-3, 35-4 and 35-5, as represented in Figure 3.
33. A nucleic acid probe of Claim 32 further comprising a polynucleotide tail.

-40-

34. A nucleic acid probe of Claim 33 wherein the polynucleotide tail is poly d(A) or poly d(T).
35. A nucleic acid probe consisting essentially of a nucleotide sequence complementary to all or a portion of the E6 gene of a HPV type selected from the group consisting of HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 42, HPV 43, HPV 44, HPV 45, HPV 51, HPV 52 and HPV 56.
36. A nucleic acid probe consisting essentially of a nucleotide sequence complementary to all or a portion of the E7 gene of a HPV type selected from the group consisting of HPV 6, HPV 11, HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 42, HPV 43, HPV 44, HPV 45, HPV 51, HPV 52 and HPV 56.
37. A nucleic acid probe comprising a nucleotide sequence complementary to the nucleotide sequence of a spliced transcript of at least a region of the E6 gene and at least a region of the E7 gene of a high-oncogenic type HPV.
38. A nucleic acid probe of Claim 37 selected from the group consisting of: ExHPV16E6*I, ExHPV16E6*II, ExHPV18E6*, ExHPV31E6*, ExHPV33E6*, and ExHPV35E6*, as represented in Figure 4.

1/8

FIGURE 1



2/8

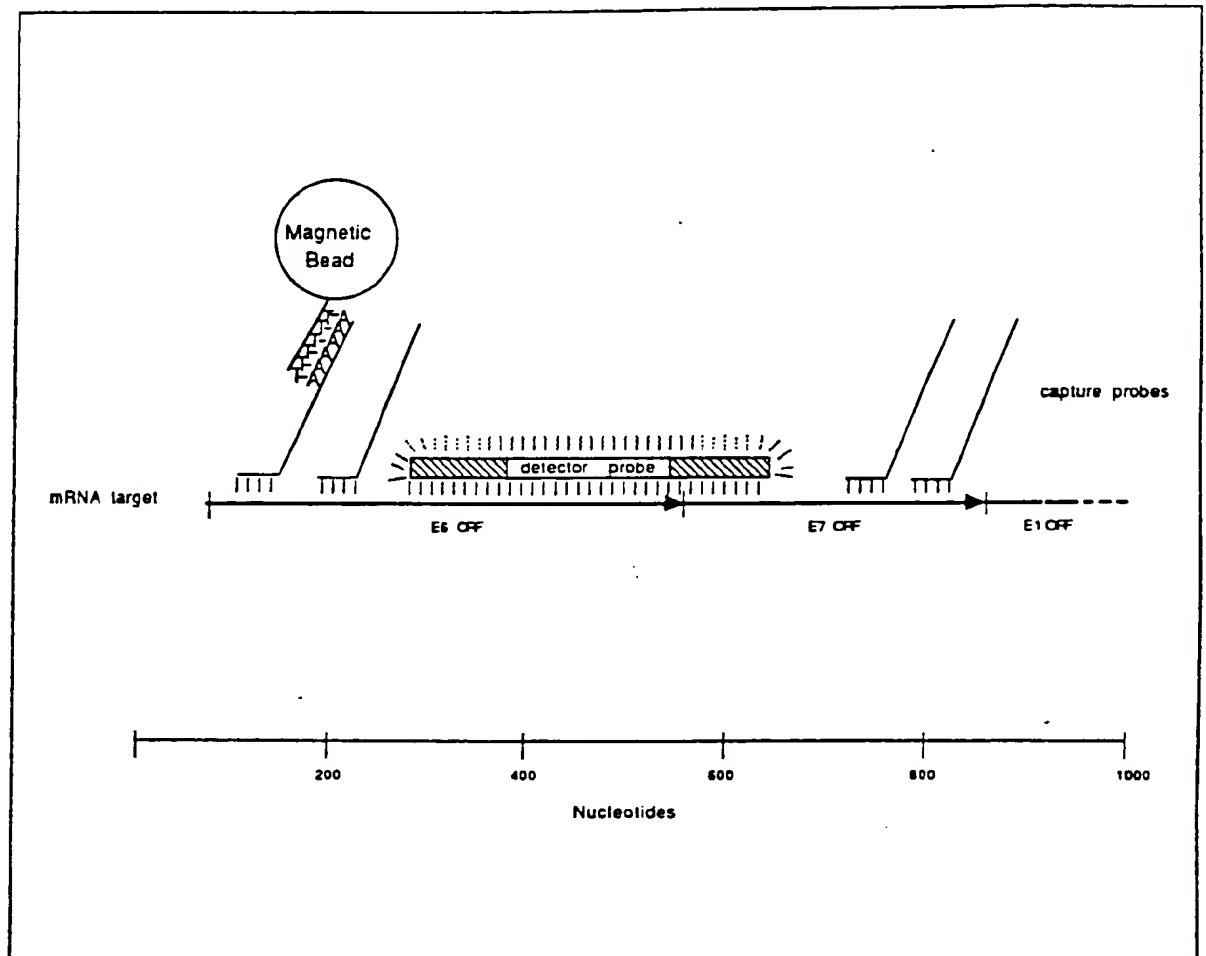


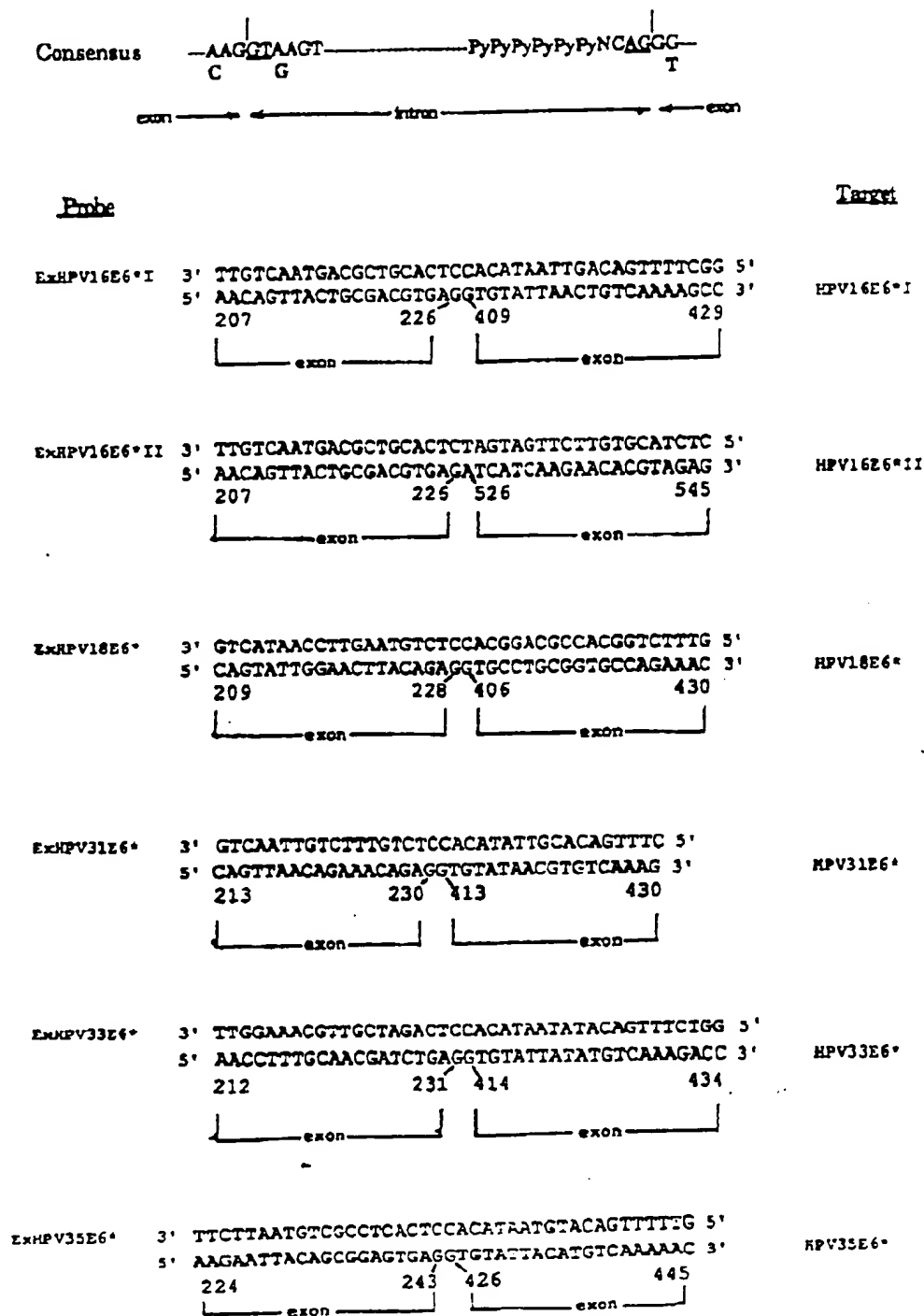
FIGURE 2

FIGURE 3

HPV	Probe No.	Oligonucleotide Sequence				Target Nts.
		5'			3'	
6	6-1	ACAACTGGTC	TATGGTCGTT	GCAGACGTGG	AGGCATTGTC	111-150
	6-2	TTGCAAAACA	CACAATTAAT	TTGCAACGTA	TGCATAGAT	167-205
	6-3	TTTAAAGGTT	GTGAATCTTG	TCCGTCCACT	TC	644-675
	6-4	GTTTCTGTAC	ACTGCACAAC	CAGTCGAACG	TTGCTG	715-750
	6-5	TATGTTTAGT	GTTOCCAACA	GAAGCTGTTG	CACTTC	761-796
11	11-1	CTCAACTGGT	CTATAGATGT	TGCAGACGTG	GAGGCATCTT	112-151
	11-2	CTGAATTTGC	AGAGTGTGCA	AAGAAAGATT	AAACGTCTTG	152-191
	11-3	TGTAACCCCTA	CAGGGTCAGG	AGGCTGCAGG	TCTAGTACTA	561-600
	11-4	CCACAGCAAC	AGGTCAGTAT	TTGGTAATGT	TGTGTTAAAG	669-708
	11-5	CCGTCTGTGC	ACTOCACAAC	CAGTCGGACG	TTGCTG	715-750
	11-6	TATTTAGTGT	GCCCAGCAAA	AGGTCTTGTA	GTTGTCTGAT	755-794
16	16-1	TCTGGGTGCG	TOCTGTGGGT	CCTGAAACAT	TGCAGTTCAT AT	96-133
	16-2	TCTAATATTA	TATCATGTAT	AGTTGTTTGC	AGCTCTGTGC	150-189
	16-3	TTGCAACAAA	AGGTTACAAT	ATTGTAATGG	GCTCTGTCCG	701-740
	16-4	GTCTACGTGT	GTGCTTTGTA	CGCACAACCG	AAGCGTAGAG	747-786
	16-5	TTCTAGTGT	GCCCATTAAAC	AGGTCTTCCA	AAGTACGAAT	787-826
18	18-1	GTAGGGTGGC	CGTGTGGAT	CCTCAAAGCG	CGCCATAG	103-140
	18-2	GTTATTTCTA	TGTCITGCG	TGAAGTGTTT	AGTTCOGTGC	157-196
	18-3	TAGAAGGTCA	ACCGGAATTT	CATTTTGGGG	CTCTAAATGC A	627-667
	18-4	TTACAACACA	TACACAACAT	TGTGTGACGT	TGTGGTTC	752-789
	18-5	GTGCTCTGCT	GAGCTTTCTA	CTACTAGCTC	AATCTGGCT	796-835
	18-6	AGGACAGGGT	GTTCAGAAAC	AGCTGCTGGA	ATGCTCGAAG	836-875
31	31-1	CAATTTCCGA	GGTCTTTCTG	CAGGATTTTT	GAACATGGCG	103-142
	31-2	GTTTCATCGTA	GGGTATTTCC	AATGCCGAGC	TTAGTTCATG	145-183
	31-3	ACGAAGTGTA	GACTTACACT	GACAACAAAA	GGTAACG	721-757
	31-4	CTCTTGCAAT	ATGCGAATAT	CTACTTGTGT	GCTCTGTACA	763-802
	31-5	CAGTCTAGTA	GAACAGTTGG	GGCACACGAT	TCCAAATGA	815-853
33	33-1	ATGCTTGGCA	CAAATCATGC	AATGTTGCTG	GTTTTTCCTC	124-163
	33-2	TTCCACGCAC	TGTAGTTCAA	TGTTGTGTAT	AGTTGTCTCC A	164-204
	33-3	GTATAGGTCA	GTTGGTTCAG	GATATAAATC	TAAACATAT	602-641
	33-4	AGTGTGACAA	CAGGTTACAA	TGTAGTAATC	AGCTGTGGCT	713-752
	33-5	TTGCTGTACT	GTTGACACAT	AAACGAACTG	TGGTGT	756-792
	33-6	TATTCAGTGT	GCCATAAGT	AGTTGCTGTA	TGGTTCGTAG	798-837
35	35-1	TAAGGTCGTT	CAGCTGGGTC	CTGAAACATA	CCGCACCTT	111-149
	35-2	CATGGATGCT	TTCTTCTACC	TCGTTGCACA	AATCATGCAG T	153-193
	35-3	CACAGACGTA	GTGTGCGCTC	ACATTTACAA	CAGGACGTT	740-778
	35-4	ATCTTCCAAT	TTACGTATGT	CAATGTGTGT	GCTCTGTACT	780-818
	35-5	CTCTCTGTGA	ACAGCCGGGG	CACACTATTC	CAAATGTGCT	829-867

FIGURE 4

Sequences and Target Sites of Splice-Junction Probes for HPV16,
HPV 18, HPV 31, HPV 33, and HPV 35



5/8

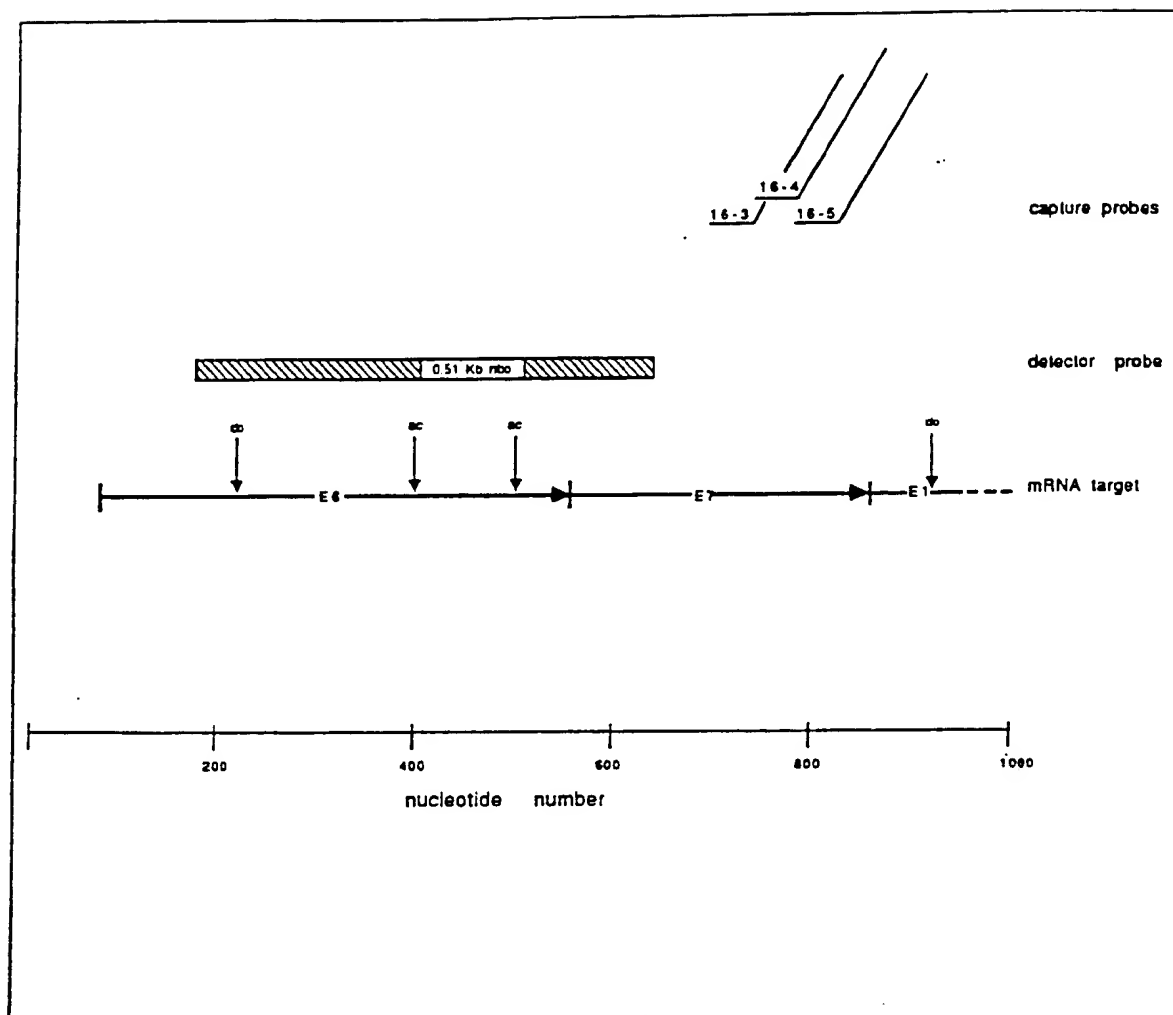


FIGURE 5

6/8

PROBE 16-3 3' GCCTGTCTCGGGTAATGTTATAACATTGGAAACAACGTT 5'
 HPV 16 5' CGGACAGAGCCCATTACAATATTGTAACCTTTTGTGCAA 3'
 nt 701 740
 HPV 6 CtttaAaAcaaCATTtCcAaATaGTgACCTgTTGcTgtgg
 HPV 11 CtttaAcAcaaCATTACcAaATactgACCTgTTGcTgtgg
 HPV 18 gaaccAcAacgtcacACAATgTTGTgtatgTgtgTaaagt
 HPV 31 CGGACAcAtCCaATTACAATATcGTtACCTTTTGTGTgcA
 HPV 33 CAGcCacAGCtgATTACtAcATTGTAACCTgTTGTcaCac
 HPV 35 CaGACAcctCCaATTAtAATATTGTAACgTcctGTTGTAA

Probe 16-4 3' GAGATGCGAAGCCAACACGCATGTTTCGTGTGTGCATCTG 5'
 HPV 16 5' CTCTACGCTTCGGTTGTGCGTACAAAGCACAACGTAAGAC 3'
 nt 747 786
 HPV 6 CagcAacgTTCGactGgttGTgCagtGtACAgAaacAGAC
 HPV 11 CagcAacgTcCGactGgttGTggAgtGCACAgACGgAGAC
 HPV 18 agCcAgaaTTgaGcTagtaGTAgAAAGCtCagcaGacGAC
 HPV 31 gTCTACaCTTCGtTTGTGtGTACAgAGCACACAaGTAGat
 HPV 33 caCcACagTTCGtTTaTGtGTcaAcAGtACAgcaagtGac
 HPV 35 ggCgACaCTaCGtcTGTGtGTACAgAGCACACaTtGAC

Probe 16-5 3' TAAGCATGAAACCTTCTGGACAATTACCCGTGTGATCCTT 5'
 HPV 16 5' ATTCTACTTTGGAAGACCTGTTAATGGGCACACTAGGAA 3'
 nt 787 826
 HPV 6 ATcaGagaagTGcAacAgCTtcTgtTGGGaAaCACTAAaca
 HPV 11 ATcaGacaacTAcAAGACCTttTgcTGGGCACACTAaatA
 HPV 18 cTTcGagcaTTccAgcAgCTGTTtcTGaacAGcCTgtccT
 HPV 31 ATTcGcAtaTTGCAAGAgCTGTTAATGGGcTgAtTtGGAA
 HPV 33 cTaCGaACcaTAcAgcAaCTacTtATGGGCACAgTgaatA
 HPV 35 ATaCGTAaaTTGGAAGattTaTTAATGGGCACAtTtGGAA

FIGURE 6

7/8

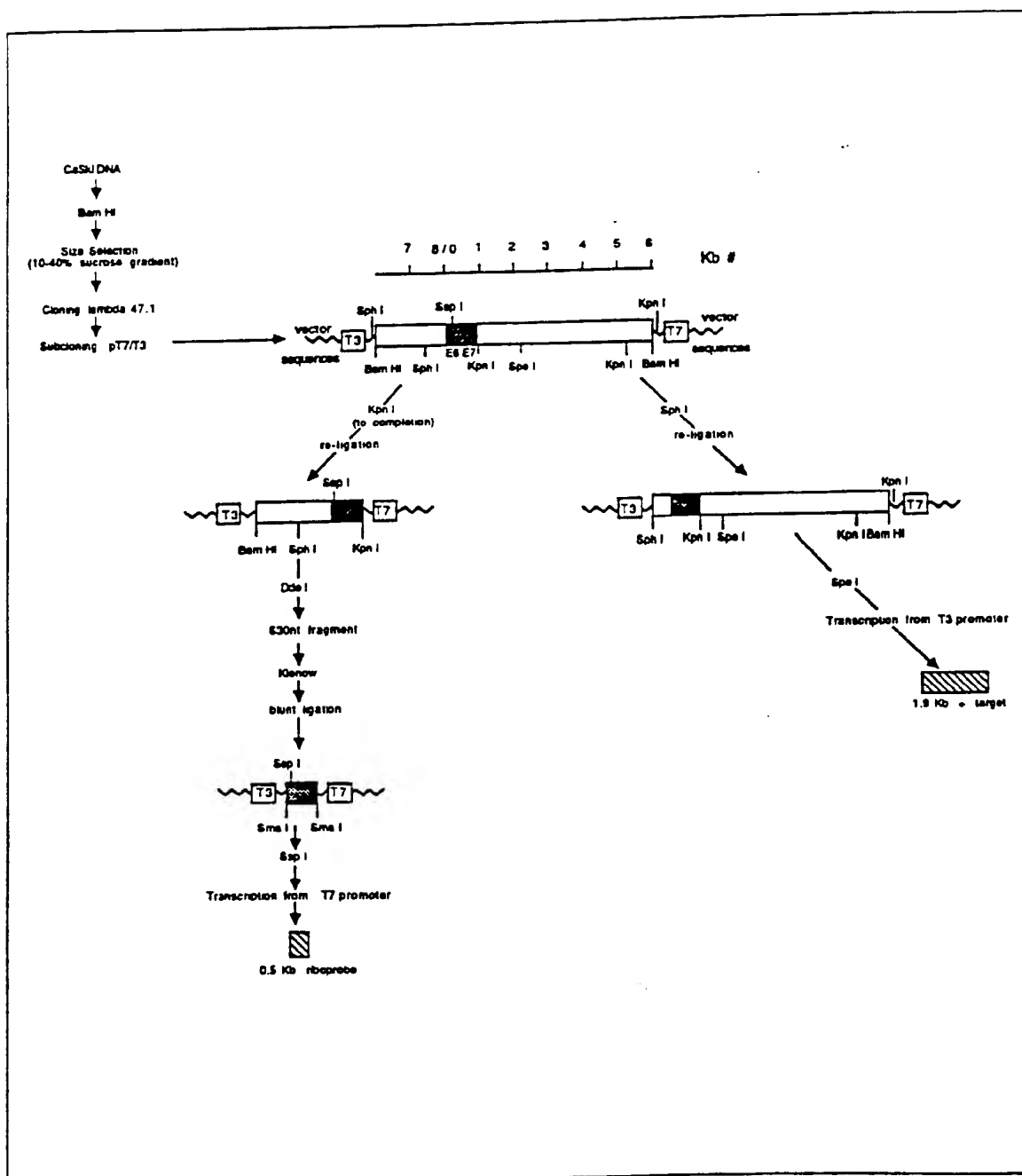


FIGURE 7

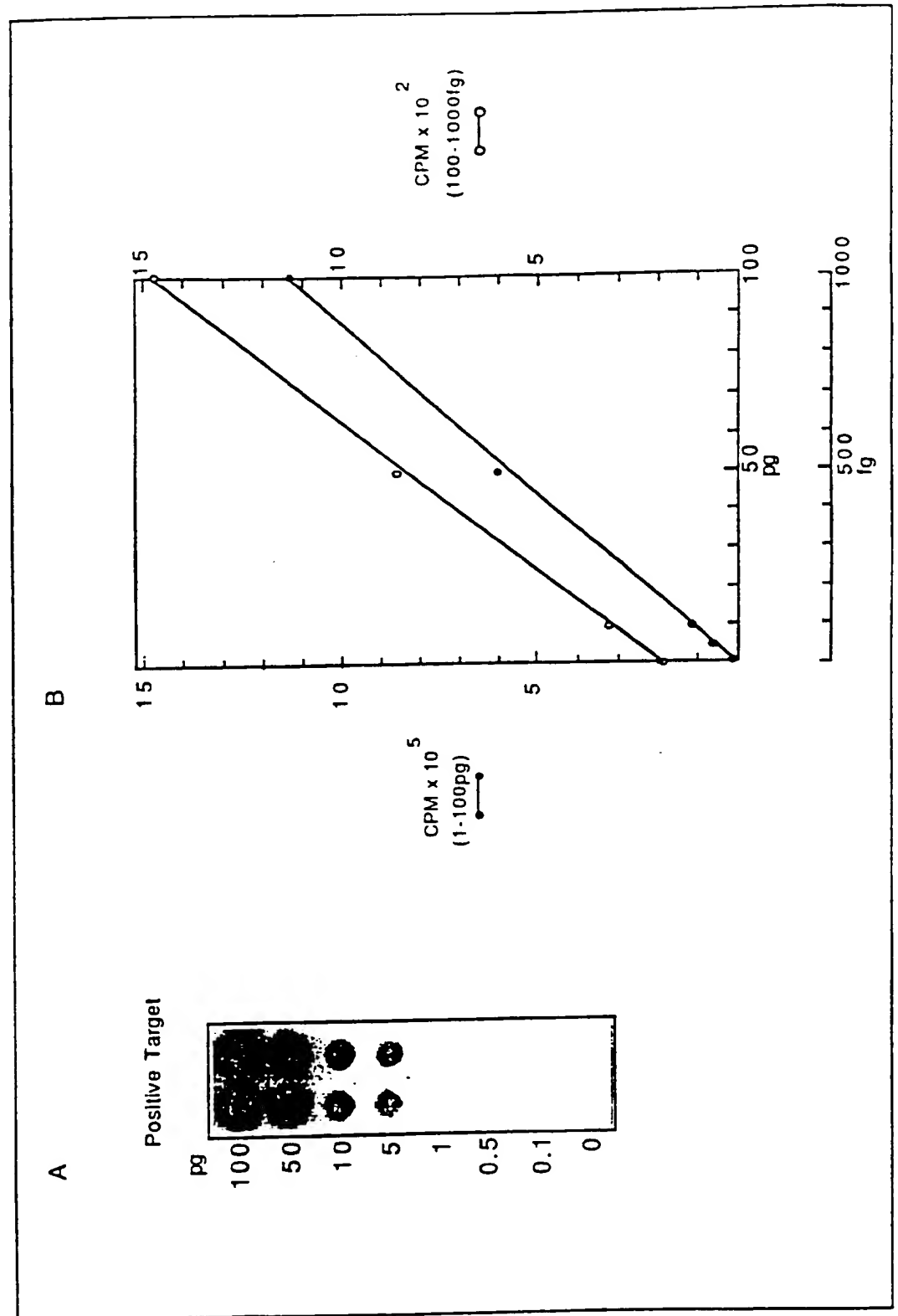


FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07057

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 Q 1/70//C 12 N 15/37														
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification System</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification Symbols</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">IPC5</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">C 12 Q</div> </td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched⁸</div>			<div style="border: 1px solid black; padding: 2px;">Classification System</div>	<div style="border: 1px solid black; padding: 2px;">Classification Symbols</div>	<div style="border: 1px solid black; padding: 2px;">IPC5</div>	<div style="border: 1px solid black; padding: 2px;">C 12 Q</div>								
<div style="border: 1px solid black; padding: 2px;">Classification System</div>	<div style="border: 1px solid black; padding: 2px;">Classification Symbols</div>													
<div style="border: 1px solid black; padding: 2px;">IPC5</div>	<div style="border: 1px solid black; padding: 2px;">C 12 Q</div>													
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th style="width: 10%;">Category[*]</th> <th style="width: 60%;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">E</td> <td>EP, A2, 0402132 (TAKARA SHUZO CO. LTD) 12 December 1990, see e.g. p.5, lines 22-28 --</td> <td style="vertical-align: top;">1-10, 13- 26, 30- 31, 33- 37</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">P, X</td> <td>EP, A2, 0373352 (BEHRINGWERKE) 20 June 1990, see e.g. p.3, lines 1-4, figures 4a, 4b --</td> <td style="vertical-align: top;">1-10, 13- 26, 30- 31, 33- 37</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">P, X</td> <td>WO, A1, 9002821 (CETUS CORPORATION) 22 March 1990, see e.g. p.21, lines 15-24 --</td> <td style="vertical-align: top;">1-10, 13- 26, 30- 31, 33- 37</td> </tr> </tbody> </table>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	E	EP, A2, 0402132 (TAKARA SHUZO CO. LTD) 12 December 1990, see e.g. p.5, lines 22-28 --	1-10, 13- 26, 30- 31, 33- 37	P, X	EP, A2, 0373352 (BEHRINGWERKE) 20 June 1990, see e.g. p.3, lines 1-4, figures 4a, 4b --	1-10, 13- 26, 30- 31, 33- 37	P, X	WO, A1, 9002821 (CETUS CORPORATION) 22 March 1990, see e.g. p.21, lines 15-24 --	1-10, 13- 26, 30- 31, 33- 37
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
E	EP, A2, 0402132 (TAKARA SHUZO CO. LTD) 12 December 1990, see e.g. p.5, lines 22-28 --	1-10, 13- 26, 30- 31, 33- 37												
P, X	EP, A2, 0373352 (BEHRINGWERKE) 20 June 1990, see e.g. p.3, lines 1-4, figures 4a, 4b --	1-10, 13- 26, 30- 31, 33- 37												
P, X	WO, A1, 9002821 (CETUS CORPORATION) 22 March 1990, see e.g. p.21, lines 15-24 --	1-10, 13- 26, 30- 31, 33- 37												
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">22nd March 1991</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">- 8. 04. 91</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">International Searching Authority</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px; text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px; text-align: center;"> <div style="border: 1px solid black; display: inline-block; padding: 2px;">M. PEIS</div> <div style="margin-left: 20px; font-family: cursive;">M. Pez</div> </div> </td> </tr> </table>			<div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div>	<div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div>	<div style="border: 1px solid black; padding: 2px;">22nd March 1991</div>	<div style="border: 1px solid black; padding: 2px;">- 8. 04. 91</div>	<div style="border: 1px solid black; padding: 2px;">International Searching Authority</div>	<div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div>	<div style="border: 1px solid black; padding: 2px; text-align: center;">EUROPEAN PATENT OFFICE</div>	<div style="border: 1px solid black; padding: 2px; text-align: center;"> <div style="border: 1px solid black; display: inline-block; padding: 2px;">M. PEIS</div> <div style="margin-left: 20px; font-family: cursive;">M. Pez</div> </div>				
<div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div>	<div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div>													
<div style="border: 1px solid black; padding: 2px;">22nd March 1991</div>	<div style="border: 1px solid black; padding: 2px;">- 8. 04. 91</div>													
<div style="border: 1px solid black; padding: 2px;">International Searching Authority</div>	<div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div>													
<div style="border: 1px solid black; padding: 2px; text-align: center;">EUROPEAN PATENT OFFICE</div>	<div style="border: 1px solid black; padding: 2px; text-align: center;"> <div style="border: 1px solid black; display: inline-block; padding: 2px;">M. PEIS</div> <div style="margin-left: 20px; font-family: cursive;">M. Pez</div> </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	WO, A1, 8909940 (ONCOR, INC.) 19 October 1989, see e.g. p.16, lines 1-18 --	1-10,13- 26,30- 31,33- 37
X	US, A, 4849332 (ATTILA T. LORINCZ) 18 July 1989, see the whole document --	1-10,13- 26,30- 31,33- 37
X	EP, A1, 0301968 (IRE-CELLTARG S.A.) 1 February 1989, see the whole document --	1-10,13- 26,30- 31,33- 37
X	WO, A1, 8806634 (THE UNIVERSITY OF SYDNEY) 7 September 1988, see the whole document --	1-10,13- 26,30- 31,33- 37
X	EP, A1, 0256321 (BEHRINGWERKE) 24 February 1988, see the whole document --	1-10,13- 26,30- 31,33- 37
X	WO, A1, 8705630 (INSTITUT PASTEUR) 24 September 1987, see the whole document --	1-10,13- 26,30- 31,33- 37
X	EP, A2, 0192001 (INSTITUT PASTEUR) 27 August 1986, see the whole document --	1-10,13- 26,30- 31,33- 37

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	J.Exp.Med., vol. 167, January 1988, Darryl K. Shibata et al.: "Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction ", see page 225 - page 230	1-10,13-26,30-31,33-37
	--	
X	J.Mol. biol., vol. 193, 1987, S.T. Cole et al.: "Nucleotide Sequence and Comparative analysis of the Human Papillomavirus Type 18 Genome ", see page 599 - page 608	1-10,13-26,30-31,33-37
	--	
X	Bull.Cancer, vol. 74, 1987, M. Ionesco et al.: "Aspects histologiques des lésions du col utérin corréles aux différents types de papillomavirus humains ", see page 397 - page 406	1-10,13-26,30-31,33-37
	--	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 27-29 because they relate to subject matter not required to be searched by this Authority, namely:

Diagnostic methods for the human or animal body C.f. PCT
rule 39 iv.

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/07057

SA 43149

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/02/91
The European Patent office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0402132	12/12/90	NONE	
EP-A2- 0373352	20/06/90	AU-D- 4460489 DE-A- 3838269 JP-A- 2187000	17/05/90 17/05/90 23/07/90
WO-A1- 9002821	22/03/90	AU-D- 4401189	02/04/90
WO-A1- 8909940	19/10/89	EP-A- 0408658	23/01/91
US-A- 4849332	18/07/89	EP-A- 0294659 JP-A- 1047383	14/12/88 21/02/89
EP-A1- 0301968	01/02/89	FR-A- 2618782 FR-A- 2629458 JP-A- 1128800 US-A- 4983728	03/02/89 06/10/89 22/05/89 08/01/91
WO-A1- 8806634	07/09/88	AU-D- 1393888 EP-A- 0357611	26/09/88 14/03/90
EP-A1- 0256321	24/02/88	AU-B- 597698 AU-D- 7601887 DE-A- 3625257 JP-A- 63214181	07/06/90 28/01/88 04/02/88 06/09/88
WO-A1- 8705630	24/09/87	AU-D- 7200787 EP-A- 0243221 JP-T- 63502798	09/10/87 28/10/87 20/10/88
EP-A2- 0192001	27/08/86	FR-A-B- 2578267 JP-A- 61216700 FR-A-B- 2581655	05/09/86 26/09/86 14/11/86

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

